

Uptake and toxicity of arsenic: *Bryum dichotomum* Hedw. — a case study

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Abstract

Bryophytes have been used widely as biomonitors to map distribution of pollutant concentrations for many years, but their reliability has been questioned. One issue was the variability of uptake capacity within a species. Many potential reasons have been suggested for this, both intrinsic and extrinsic. This paper provides a brief review of arsenic uptake and tolerance within plants, particularly bryophytes, and examines the variability in uptake of arsenic using *Bryum dichotomum* Hedw., a moss, as a model organism. Samples were used from two sites, one with low and one with high arsenic emission levels. Differences in uptake were noted and it is suggested that these differences are the result of acclimation to exposure levels at the site from which they were removed. This acclimation could be simple hardening common to many plants or genetic differentiation into ecotypes. The findings of this study have implications with regards to the suitability of bryophytes as biomonitors of metal pollution. (*The Victorian Naturalist* 131(6), 2014, 192-203)

Key words: *Bryum dichotomum*, arsenic, biomonitor, metal uptake, metal pollution

Introduction

The element arsenic, is ranked 20 in abundance in the earth's crust and its presence considered both a major health concern and pollutant on a global scale (Cullen and Reimer 1989; McArthur 1999). In the past, arsenic was used in a number of agricultural pesticides. Understandably, therefore, some of the first investigations into the effects of arsenic on plants were carried out on crop plants in an endeavour to determine whether crops sprayed with pesticides containing arsenic would affect those consuming them (Murphy and Aucott 1998; Wells and Gilmour 1977; Woolson *et al.* 1971). These studies determined that certain crop plants retained arsenic above and beyond that found in the soil. Because of this, pesticides that contained lead arsenate were banned in most developed countries during the 1980s (Peryea 1998) and have since been replaced with herbicides containing the less toxic form monosodium methyl arsenate (World Health Organisation (WHO) 2005).

Interest in the tolerance of plants to arsenic has continued to grow, especially because tens of millions of people are exposed to high levels of arsenic in West Asia through the continued consumption of contaminated food plants (Roychowdhury *et al.* 2003; Roychowdhury *et*

al. 2002). The uptake of arsenic by plants may have caused concern for the public health because of contaminated food sources, but it also provided a possible means of removing arsenic from contaminated environments. While the research on use of plants as a remediator for arsenic contaminated soils started back in the 1970s, more recently (2000 to 2009) a wealth of studies have been published (Table 1), although only few on bryophytes. These studies have shown that hyperaccumulators can be found throughout the plant kingdom and include flowering plants, ferns and bryophytes. Plant species vary in their capacity to take up arsenic, and taxonomic affinity does not necessarily translate to similar uptake abilities. For example, the fern species *Pteris vittata* can hyperaccumulate 3894 µg of arsenic per gram of dried plant material (µg/g) (Zhang *et al.* 2002), but the congeneric species *Pteris tremula* and *Pteris straminea* can take up only 16.6 and 78.0 µg/g respectively (Ma *et al.* 2001; Meharg 2003). Koch *et al.* (1999) examined arsenic content of a wide variety of plants and found that mosses contained the highest levels per unit of dried weight but species varied in uptake capacity, 490–1229 ppm dry weight. Floyed (2010) also showed this variation in uptake capacity, as

Table 1. Examples of studies investigating arsenic in various plant groups.

Authors	Plant groups investigated	number of species	Area of study
Samecka-Cymerman and Kempers (1994)	Bryophytes	5	Bioindication
Koch <i>et al.</i> (1999)	Algae	2	Biomonitoring
	Bryophytes	1	
	Lichens	4	
	Fungi	3	
	Flowering plants	41	
Koch <i>et al.</i> (2000)	Bryophytes	1	Arsenic speciation
	Flowering plants	12	
Ma <i>et al.</i> (2001)	Ferns	1	Hyperaccumulation
Francesconi <i>et al.</i> (2002)	Ferns	1	Hyperaccumulation
Lombi <i>et al.</i> (2002)	Ferns	1	Arsenic distribution and speciation within fronds
Visoottiviset <i>et al.</i> (2002)	Grasses	4	Accumulation/hyperaccumulation
	Flowering plants	21	
	Ferns	6	
	Trees	521	
Zhang <i>et al.</i> (2002)	Ferns	1	Arsenic speciation and distribution within plants
Zhao <i>et al.</i> (2002)	Ferns	11	Hyperaccumulation
Aceto <i>et al.</i> (2003)	Bryophytes	1	Bioindication
Meharg (2003)	Ferns	45	Accumulation/hyperaccumulation
	Fern allies	45	
Robinson <i>et al.</i> (2003)	Flowering plant	1	Uptake
Salido <i>et al.</i> (2003)	Ferns	1	Phytoremediation
	Flowering plants	1	
Warren <i>et al.</i> (2003)	Flowering plants	6	Uptake
Zhang <i>et al.</i> (2004)	Ferns	1	Characterisation of arsenic uptake
Duan <i>et al.</i> (2005)	Ferns	1	Characterisation of arsenic uptake
Fayiga and Ma (2005)	Ferns	2	Uptake
Robinson <i>et al.</i> (2006)	Ferns	5	Hyperaccumulation
	Flowering plants	5	
Van <i>et al.</i> (2006)	Ferns	1	Accumulation
Wei and Chen (2006)	Ferns	2	Accumulation
Catarecha <i>et al.</i> (2007)	Flowering plants	1	Accumulation
Craw <i>et al.</i> (2007)	Bryophytes	4	Accumulation
	Ferns	4	
	Flowering plants	12	
Shahraki <i>et al.</i> (2008)	Flowering plants	5	Phytoremediation

have other studies. Koch *et al.* (1999), Aceto *et al.* (2003) and Craw *et al.* (2007) found *Funaria hygrometrica*, *Bryum argenteum* and *Pohlia wahlenbergii* respectively had arsenic compositions up to 350 µg/g, 10.9 µg/g and 29 000 µg/g, the latter value being over the hyperaccumulator threshold of 1000 µg/g.

Coping mechanisms for arsenic tolerance varies. Some vascular species such as the tomato *Lycopersicon esculentum* Mill. var. *esculentum*, store arsenic within their root system (Carbonell-Barrachina *et al.* 1997), while other species, such as *Pteris vittata*, transport arsenic

from the roots to the shoots where it is stored (Zhang *et al.* 2002). The storing of arsenic in the roots is considered a sign of arsenic exclusion (Carbonell-Barrachina *et al.* 1997), while the translocation of arsenic from the roots to the shoots, especially to senescing leaves, is seen as a means of detoxification as arsenic is removed from the plant at leaf fall (Dahmani-Muller *et al.* 2000). In yet other fern species, it appears that arsenic is actively removed via translocation from the senescent frond to younger fronds (Francesconi *et al.* 2002).

Within marine organisms, arsenic is normally found in organic forms such as arsenosugars in algae, and arsenobetaine and arsenocholine in fish, molluscs and crustaceans (Francesconi *et al.* 1994; Maeda 1994). In vascular plants, arsenic is normally stored as the more toxic inorganic forms of arsenate (As[V]) and arsenite (As[III]) (Koch *et al.* 1999; Koch *et al.* 2000; Zhang *et al.* 2002). Because of the chemical similarities of arsenate and phosphate, arsenic competes against phosphate for the phosphate uptake system (Macnair and Cumbes 1987; Meharg and Macnair 1990, 1991; Wells and Richardson 1985), and is taken up through vascular plant root systems as arsenate (Zhang *et al.* 2002). Once arsenate has entered the plant, it is reduced to arsenite as a means of detoxification within the plant (Zhang *et al.* 2002). Arsenite, while more toxic, is bound to ligands (or chelators) and then can be compartmentalised in the vacuoles which help stabilise the complexes due to their acidic nature, thereby avoiding damage to the cells (Meharg and Hartley-Whitaker 2002). Thus, as long as the samples are treated so that both arsenate and arsenite may be measured, a true indication of arsenic content may be achieved.

Certain bacteria and yeasts reduce arsenate to arsenite, and can efflux arsenite from their cells through transporters (Rosen 1999). *Saccharomyces cerevisiae* also can form complexes between arsenite and glutathione which then can be actively transported into the vacuole through a specialised transporter (Rosen 1999). It is speculated that the arsenite is bound to phytochelatins which are transported into the vacuole (Meharg and Hartley-Whitaker 2002). While arsenic phytochelatins are not stable under either neutral or alkaline conditions, they are stable under acidic conditions, which normally are found within the vacuole (Schmoger *et al.* 2000; Sneller *et al.* 2000).

Uptake and toxicity studies can resolve a number of issues. For example, they may determine the sensitivity of a species to the element in question by determination of its lethal dose; they may help to determine if species will react in a progressive manner to a particular substance under sequential concentration loads; they can determine if reactions vary based on where samples originated. These are important

questions that should be answered with respect to a species that is used as a biomonitor as it may help explain the sometimes confounding results of fieldwork. Lichens acclimated to different concentrations of an element are well known to display different sensitivities to that element (Bennett 2002; Freitas *et al.* 1999; Herzig 1993; LeBlanc *et al.* 1972; Loppi and Bonini 2000; Nieboer *et al.* 1977; Reis *et al.* 2002; Seaward 1995). This means one cannot simply infer that the behaviour of a species in one area reflects the behaviour of the same species in another area. The same concept generally is applicable to plants, which undergo the process of 'hardening' to become acclimated to changed conditions (Raven *et al.* 1992). This is independent of the findings of Shaw (1994) who postulated that different ecotypes of a species evolve as a response to natural selection in contaminated sites, over a few years, resulting in a species genetically acclimated to different pollutant levels as opposed to only physiologically acclimated. In the laboratory, it is possible to isolate effects to a single element or a specific combination of elements under controlled conditions. In the field, an organism responds to all factors it experiences, including synergistic effects. It can be useful to have an understanding of an organism's behaviour under controlled conditions to provide insights into field data.

Floyd (2010) showed that *Bryum dichotomum* was a moss common to urban streetscapes, occurring at 65 of 88 sites and during any season. Further, it occurred at 42%, 68% and 67% of the sites that released low, medium, and high levels of arsenic respectively and was identified as a hyperaccumulator, being able to accumulate up to 15 134 µg/g arsenic to plant weight (soil concentration was 409 µg/g). This suggests it has potential as a biomonitor of arsenic pollution and should be investigated further. Other species, for example, the liverwort *Chyloscyphus semiteres* var. *semiteres*, also accumulated high levels of arsenic, but were not deemed as ideal samples for further study, either because they were not widespread or because they were not present throughout the year. Uptake of arsenic by *B. dichotomum*, however, was variable and many possible reasons for this were identified. Laboratory controlled investigations help to explain this by removing external environmental

influences on uptake, thereby showing whether biology of the organism was the cause of such variation.

This study examined the behaviour of *B. dichotomum* in terms of its sensitivity to arsenic by determining whether samples acclimated to high levels of arsenic and samples acclimated to low levels of arsenic varied in:

1. the amount of arsenic they accumulated;
2. rate of uptake; and
3. cell viability when exposed to a range of arsenic concentrations.

It is hypothesised that there will be a difference in each instance based on the concept that the species can become acclimated, either physiologically or through development of ecotypes.

Method

Study sites

Bryum dichotomum was collected in the summer of 2006 from the streetscapes of two study sites within Victoria, the Commonwealth Serum Laboratories (CSL) in Parkville, which emits 0.012 kg of arsenic per year, and the Austin Hospital (Austin) in Alphington, which emits 1.1 kg of arsenic per year. The CSL is located near the centre of Melbourne, while the Austin Hospital is east/north east of the CBD. Both sites have streetscapes on all four sides and are surrounded by a combination of other businesses and residential housing. Both experience the same weather: summer – 13.9 to 25.3°C; autumn – 10.8 to 20.3°C; winter – 6.5 to 14.1°C; spring – 9.5 to 19.5°C (Australian Bureau of Meteorology (BOM): <http://www.bom.gov.au/climate>). Mean monthly rainfall for summer, autumn, winter and spring were 49.1, 47.8, 47.0 and 56.5 mm respectively.

Sampling

At each site, samples were collected from a single large colony and transported back to the laboratory where they were carefully cleaned of particulate matter with the aid of a fine paint brush and an Olympus SZ-PT dissecting microscope.

Culturing

Bryophyte toxicity to three concentrations of arsenic was tested: 100 ppb, 1000 ppb and 10000 ppb arsenic. These were standard concentrations for toxicological studies of arsenic

by the WHO (2000). A control sample for each site was exposed to double distilled water. For each test group, the following time course of exposure was conducted: 0, 1, 2, 4, 8, 12 and 24 hours after Pickering and Puia (1969) who noted that the largest amount of zinc was taken up within the first 24 hours and that at least 50% of zinc absorbed at equilibrium was done so within the first hour for the aquatic moss *Fontinalis antipyretica* L. ex. Hedw. This was replicated three times.

Ten stems of *B. dichotomum* were used per vial. Material was incubated in a Constant Temperature Cabinet using NEC Tri-phosphor 30 watt fluorescent tubes under constant lighting. Vials used for culturing were first washed manually, dried, and then treated with a two part acid wash consisting of an initial 24 hour wash in 1.2M HCl, followed by a 24 hour wash in 10% HNO₃. They were then rinsed in de-ionised water, dried and stored in sealed containers until used.

Viability testing

A total of five mature leaves were sampled from the topmost portion of stems from each culture sample, mounted onto slides and stained with Neutral Red (0.1%) to determine tissue viability. Neutral Red is taken up by the vacuole in viable cells (Fig. 1a) (Castro-Concha *et al.* 2006), thus the percentage of leaf tissue that remained viable could be determined. This process was repeated using Evans Blue (0.1%) which is excluded from viable cells by the plasmalemma (Fig. 1b) (Castro-Concha *et al.* 2006). The two stains were used to provide cross verification. A dose was determined as lethal when there was less than 50% cell viability (Trevan 1927).

Uptake

Prior to any chemical analysis being undertaken, it was essential that any equipment to be used during the acid digestion process be cleaned thoroughly to remove any possible trace metal contamination (Reeve 2002). Thus all equipment was washed as described for glassware under the section on culturing.

Once samples were removed from their respective dosages they were weighed with a Mettler AC100 digital scale and dried in a Qualtex Solidstat OG18S Gravity Convection Oven at 85°C until constant weight was achieved. The

dried material was ground into a fine powder with the aid of a mortar and pestle and transferred to 50 ml plastic centrifuge tubes containing 5 ml of concentrated Aristar HCl. Samples then were incubated in a hot water bath at 80°C for 24 hours, after which they were made up to a final volume of 20 ml by the addition of double distilled water and then centrifuged in a Clements 2000 Centrifuge at a speed of 3500 rpm for 15 minutes or until a pellet was formed. The supernatant was removed and stored in sealed containers prior to metal analysis.

Analysis of arsenic concentrations in bryophytes was performed using Hydride Generation Atomic Absorption Spectrophotometry (HG-AAS) after the method outlined by Ellis and Tyson (1996). Calibration of the HG-AAS was carried out using a series of arsenic standards (0, 5, 10 and 20 µg/ml) prepared prior to analysis.

One hour prior to analysis, 5 ml of the sample was decanted into a separate container and treated with 1 ml of 10% m/v potassium iodide solution. The addition of the potassium iodide solution reduced As^{5+} to As^{3+} allowing for the maximum arsenic response to be obtained (Barra *et al.* 2000).

Analysis of arsenic content was achieved through the method referred to as 'continuous flow technique', i.e. the sample is combined with a number of other solutions (in this case HCl, a reducing agent (0.6% NaBH_3) and 0.5% m/v

NaOH), which results in the formation of arsine gas (AsH_3). The gas is then drawn into the gas/liquid separator before being sucked into the detection cell where the absorbance of the arsenic can be calculated. In some instances the samples contained concentrations higher than could be read by the HG-AAS, so were diluted as necessary with 50% HCl.

To determine extraction efficiency, results were calibrated against those of a Standard Reference Material (SRM) 1570, spinach, with known concentrations of the five metals, and was analysed using the same protocol. This was obtained from the National Bureau of Standards (United States Department of Commerce). To determine the uptake and release of arsenic over the time course, samples were calibrated against baseline values present in *B. dichotomum* obtained prior to the exposure experiments being carried out.

Statistics

Comparisons of the uptake and viability of *B. dichotomum* collected from both sites was investigated using Analysis of Variance (ANOVA). The software package *Statistical Package for the Social Sciences* (SPSS) for windows v11 was used for these analyses. The Tukey test was applied as a *post hoc* test only where *F* values were significant. The purpose of the Tukey test is to distinguish which mean differences are significant (Fowler *et al.* 1998).

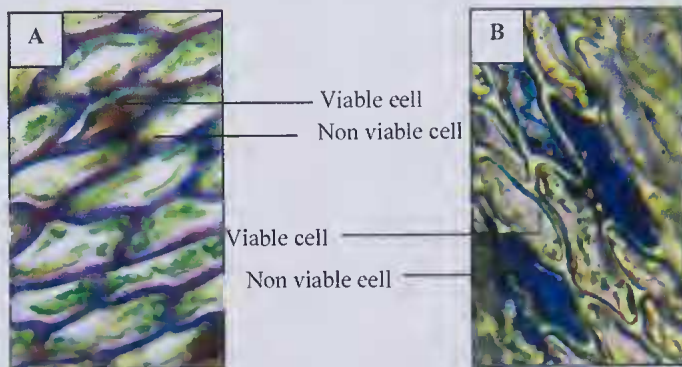


Fig. 1. Cells from leaves of *Bryum dichotomum* exposed to arsenic: A. stained with 0.1% Neutral Red. Viable cell arrowed. Neutral Red is taken up by the vacuole in viable cells B. stained with 0.1% Evans Blue. Non-viable cells arrowed. Viable cells exclude Evans Blue.

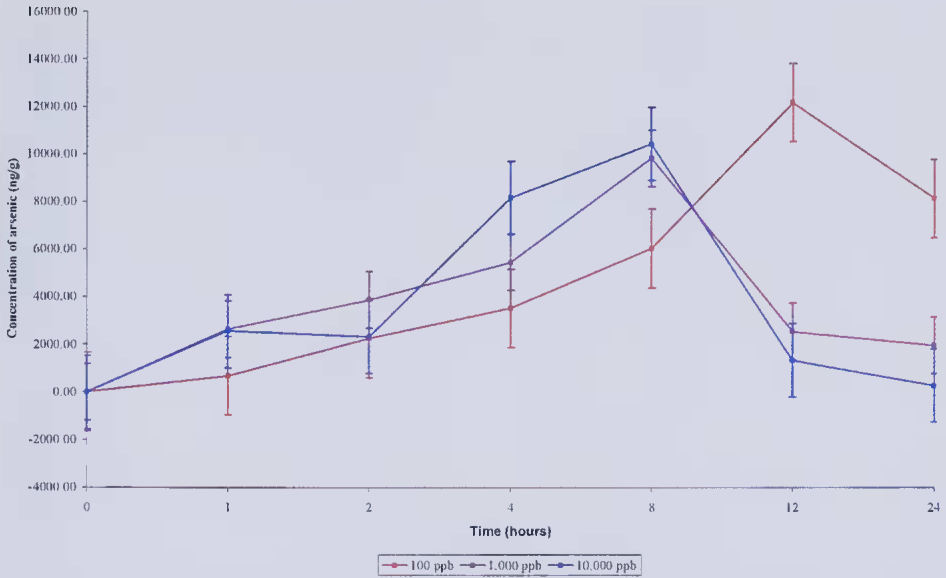


Fig. 2. Uptake of arsenic in *Bryum dichotomum* samples from Commonwealth Serum Laboratories (low arsenic) over a 24 hour exposure period.

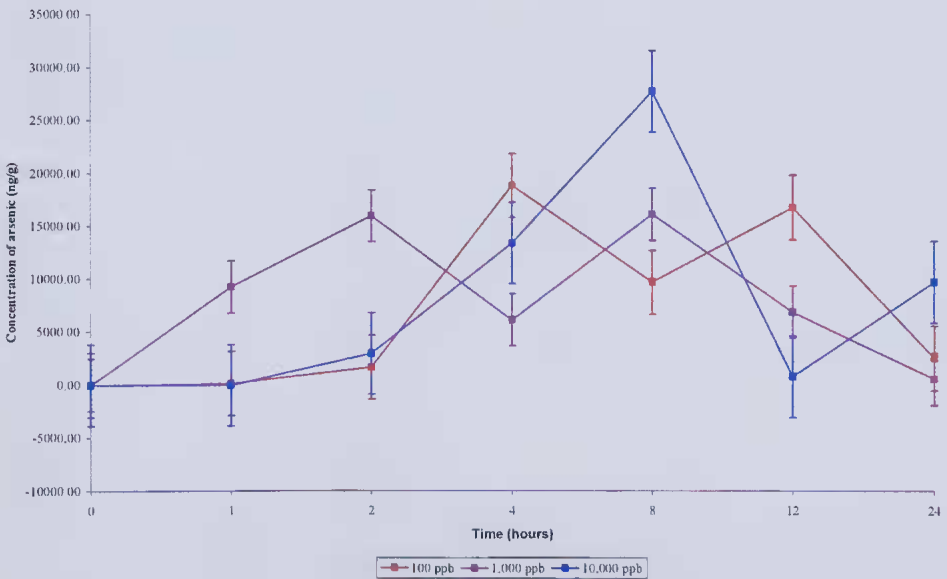


Fig. 3. Uptake of arsenic in *Bryum dichotomum* samples from Austin Hospital (high arsenic) over a 24 hour exposure period.

Results

Arsenic accumulation in *B. dichotomum* varied between the samples collected from the two areas ($F_{22,5} = 16.13$, $p < 0.003$) (Figs. 2 and 3). Samples from the site with high arsenic pollution (Austin) took up more arsenic than samples from the site with low pollution (CSL). Mean maximum arsenic levels reached 12, 9 and 10 $\mu\text{g/g}$ at 100 ppb, 1000 ppb and 10000 ppb exposures respectively in CSL samples and 19, 16.04 and 27.65 $\mu\text{g/g}$ respectively for the same exposures in the Austin samples. This is respectively 120, 9 and 10 times the exposure concentrations for CSL samples and 190, 16 and 2.7 times for Austin samples. Within sites, there was no significant difference in uptake of arsenic between exposure concentrations for either site.

The pattern of arsenic accumulation in species was the same within sites although not synchronised with time. Samples collected from CSL (low arsenic) peaked at 8 hours when exposed to solutions of 1000 and 10000 ppb and at 12 hours when exposed to 100 ppb (Fig. 2). After this, arsenic levels decreased. Arsenic levels in samples collected from Austin (high arsenic) fluctuated (Fig. 3).

Cell viability remained comparatively constant for control samples but decreased to about 60% viability after the 24 hours exposure to arsenic, with only minor differences occurring due to the exposure concentrations (Figs. 4 to 7). As expected, significant differences occurred between the control groups and the three dosages of arsenic within samples from both CSL (low arsenic) (Neutral Red $F_{27,3} = 3.561$, $P < 0.02$, Evans Blue $F_{27,3} = 3.936$, $P < 0.02$) and Austin (high arsenic) (Neutral Red $F_{27,3} = 3.219$, $P < 0.04$, Evans Blue $F_{27,3} = 2.852$, $P < 0.05$). Significant differences did not occur between the three dosages of arsenic for samples from either CSL or Austin.

Discussion

That a significant difference occurs in uptake of arsenic in samples of a species acclimated to different concentrations of that metal has serious implications for its use as a biomonitor. Many studies have mapped the distribution of air pollutants by determining the elemental concentrations within one, or more, species of

bryophyte or lichen without first testing the uptake (and release) response/s to the pollutant/s in question. Studies have estimated deposition rates of the pollutants on the presumption that these are implicitly reflected by the elemental concentration in the biomonitor species (Wolterbeek 2003), i.e. that there is a positive correlation. Certainly there are studies that indicate this is the case (e.g. Gilbert 1968; Rühling and Tyler 1973; Steinnes *et al.* 1992) but it is not always so. The results presented in this paper clearly show that samples of *B. dichotomum* acclimated to different arsenic concentrations have different uptake responses; those acclimated to high ambient arsenic had greater uptake efficiency than those acclimated to lower ambient arsenic, i.e. when samples acclimated to high arsenic levels were placed in the same ambient arsenic as samples acclimated to low arsenic levels, the former took up significantly more arsenic than the latter. Other studies also have shown such a differential response in biomonitor performance (Briggs 1972; Brown and Buck 1978; Cai and Ma 2003; Fernández and Carballeira 2000; Shaw 1994). If the difference in biomonitor response to a pollutant correlated with the change in ambient levels of that pollutant, deposition levels would be predictable and the biomonitor could be used for mapping the distribution in concentration of that pollutant. But whether this is the case must be investigated.

Other studies have shown impacts on biomonitor-moss performance due to season, e.g. Markert and Weckert (1989) for *Polytrichum formosum*; Aceto *et al.* (2003) for *B. argenteum*; and LeBlond *et al.* (2004) for *Scleropodium purum*. Åyräs *et al.* (1997) and Bargagli *et al.* (2002) also demonstrated seasonal fluctuation of element uptake by bryophytes. Other factors also affect uptake (and release) efficiencies of biomonitors, e.g. sea salt (Berg *et al.* 1995; Berg and Steinnes 1997; Gjengedal and Steinnes 1990); acidic precipitation (Gjengedal and Steinnes 1990); acid precipitation (Gjengedal and Steinnes 1990); variability in macro and microclimatic conditions of temperature, humidity, light and altitude (Seaward *et al.* 1988; Wolterbeek *et al.* 1996); phosphorous levels (Meharg and Macnair 1990); the concentration of the pollutant being examined (Kansanen and Venetvaara 1991); redistribution of metals

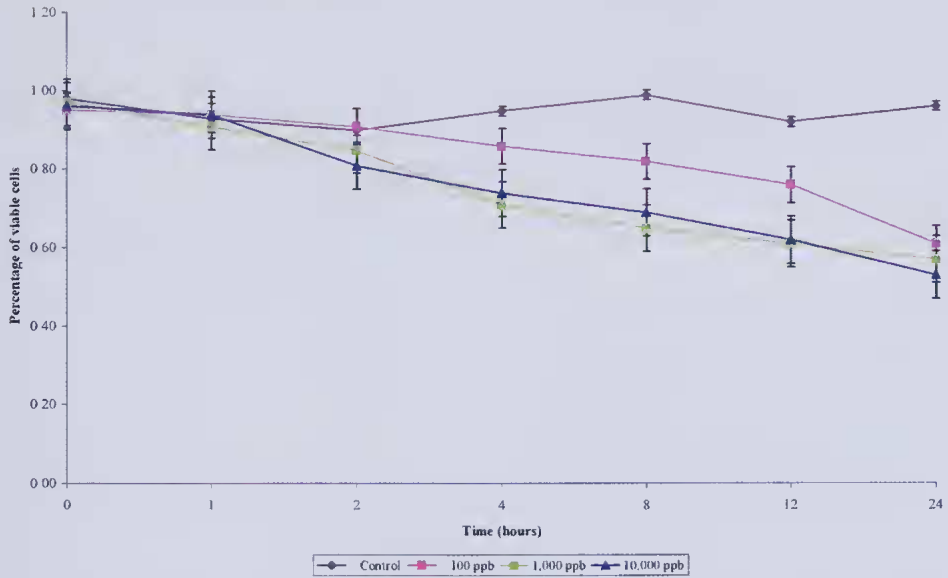


Fig. 4. Percentage of viable cells within leaves of *Bryum dichotomum* collected from CSL, a site of low arsenic pollution. Viability was determined using Neutral Red.

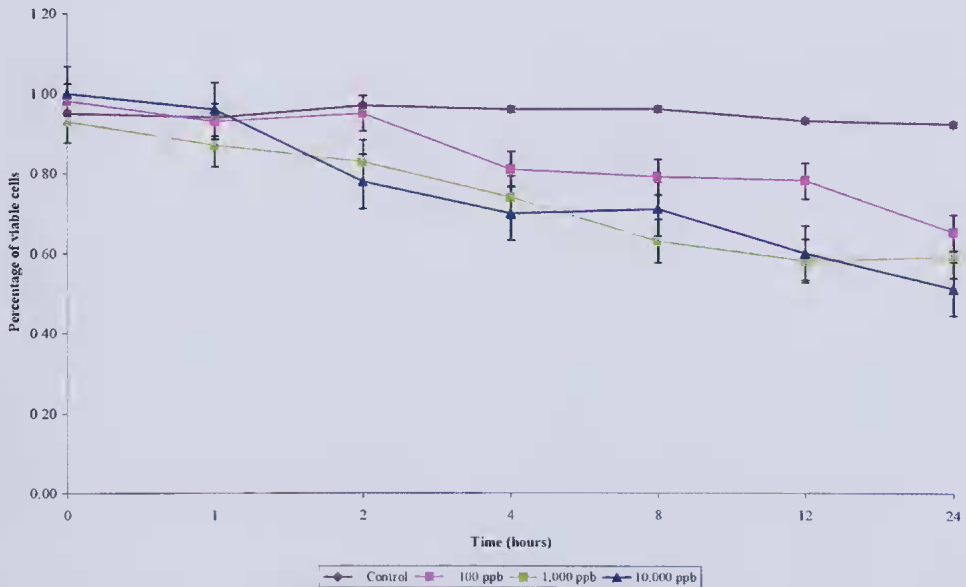


Fig. 5. Percentage of viable cells within leaves of *Bryum dichotomum* collected from CSL, a site of low arsenic pollution. Viability was determined using Evans Blue.

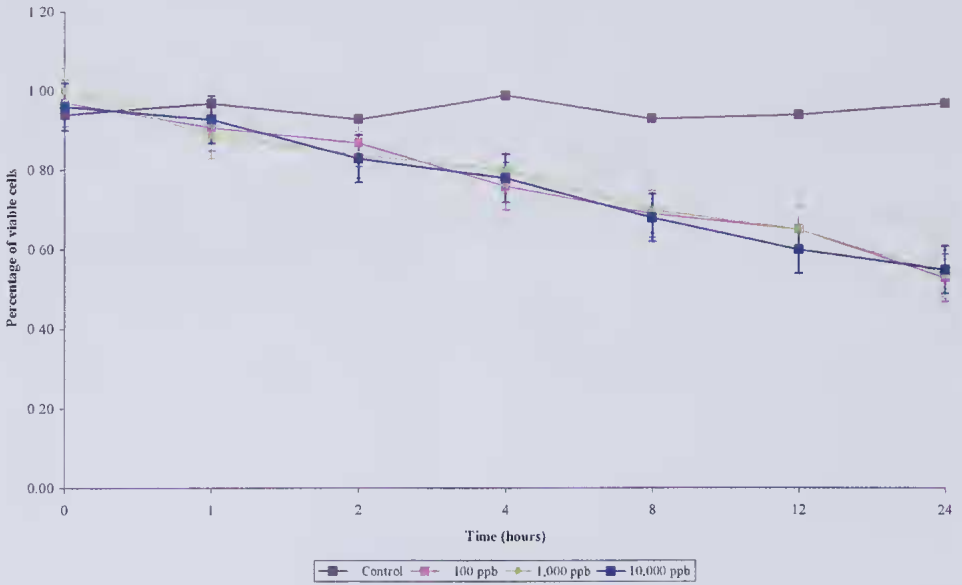


Fig. 6. Percentage of viable cells within leaves of *Bryum dichotomum* collected from Austin Hospital, a site of high arsenic pollution. Viability was determined using Neutral Red.

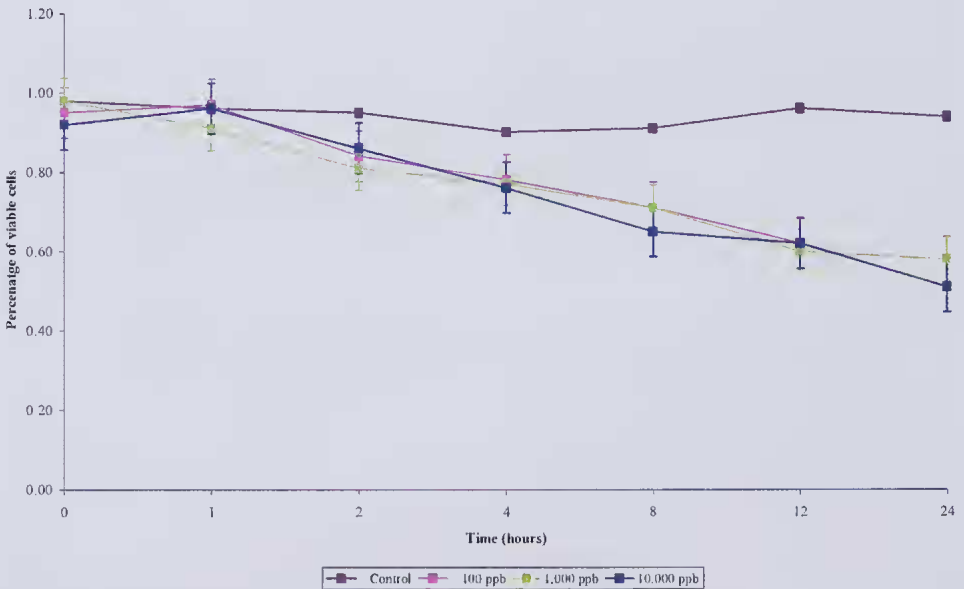


Fig. 7. Percentage of viable cells within leaves of *Bryum dichotomum* collected from Austin Hospital, a site of low arsenic pollution. Viability was determined using Evans Blue.

within a biomonitor (Brown and Wells 1990; Tyler 1990; Wells and Brown 1990); loss of metals (Sloof and Wolterbeek 1992; Taylor and Witherspoon 1972).

In consideration of the above and the results of this paper, there is a need for a combined approach in field work and laboratory analyses for pollution biomonitoring with respect to pollution mapping of actual elemental concentrations. Simple presence/absence of a pollutant can be mapped by presence of the pollutant within a biomonitor and the use of hyperaccumulators can detect habitat presence of a pollutant in minute amounts, which is important for managing potential long term effects on plant health as well as, of course, human health, but any inclusion of elemental concentrations would have to be treated with caution.

This study showed that in samples of *B. dichotomum* acclimated to low ambient arsenic, uptake rate of arsenic peaked, dropped markedly then stabilised to a low rate as indicated by the shape of the graph (Fig. 2), yet in the field this species occurs in areas with much higher concentrations of arsenic than those tested in the laboratory. Change in uptake did not match viability results, which showed a steady decrease to about 60% cell viability. Samples acclimated to the high ambient arsenic showed the same viability results over the time course of the experiment as did samples to low ambient arsenic but uptake of arsenic fluctuated. This is difficult to explain but such discrepancies in viability tests between field and laboratory data previously have been observed (Guschina and Harwood 2002; Tremper *et al.* 2004). It would have been better to have tested the arsenic levels of the incubating solution along the time series to compare with tissue solutions, but this was not done. This could have corroborated whether decrease in elemental concentration of tissue was associated with release of arsenic back into the solution. It is highly recommended that this is done in future work. It also would be useful to examine a break-down of the inter-, intra- and extra-cellular proportions of arsenic as this may affect any physiological response (Vázquez *et al.* 1999).

Concluding remarks

Variability in uptake ability of arsenic by *B. dichotomum* because of its source of origin has

important ramifications for biomonitoring applications. A species hardened to a specific environment may have a very different performance response than one hardened to another environment. Similarly, one ecotype will have a different performance response from another. Unless performance response is understood for a species, it cannot be used reliably as a biomonitor to map elemental concentrations of pollutants.

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Received 23 October 2014; accepted 20 November 2014